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Inclusion of Flagellin during Vaccination against Influenza Enhances Recall Responses in Nonhuman Primate Neonates

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ABSTRACT

Influenza virus can cause life-threatening infections in neonates and young infants. Although vaccination is a major countermeasure against influenza, current vaccines are not approved for use in infants less than 6 months of age, in part due to the weak immune response following vaccination. Thus, there is a strong need to develop new vaccines with improved efficacy for this vulnerable population. To address this issue, we established a neonatal African green monkey (AGM) nonhuman primate model that could be used to identify effective influenza vaccine approaches for use in young infants. We assessed the ability of flagellin, a Toll-like receptor 5 (TLR5) agonist, to serve as an effective adjuvant in this at-risk population. Four- to 6-day-old AGMs were primed and boosted with inactivated PR8 influenza virus (IPR8) adjuvanted with either wild-type flagellin or inactive flagellin with a mutation at position 229 (m229), the latter of which is incapable of signaling through TLR5. Increased IgG responses were observed following a boost, as well as at early times after challenge, in infants vaccinated with flagellin-adjuvanted IPR8. Inclusion of flagellin during vaccination also resulted in a significantly increased number of influenza virus-specific T cells following challenge compared to the number in infants vaccinated with the m229 adjuvant. Finally, following challenge infants vaccinated with IPR8 plus flagellin exhibited a reduced pathology in the lungs compared to that in infants that received IPR8 plus m229. This study provides the first evidence of flagellin-mediated enhancement of vaccine responses in nonhuman primate neonates.

IMPORTANCE

Young infants are particularly susceptible to severe disease as a result of influenza virus infection. Compounding this is the lack of effective vaccines for use in this vulnerable population. Here we describe a vaccine approach that results in improved immune responses and protection in young infants. Incorporation of flagellin during vaccination resulted in increased antibody and T cell responses together with reduced disease following virus infection. These results suggest that flagellin may serve as an effective adjuvant for vaccines targeted to this vulnerable population.

Influenza virus remains one of the leading causes of morbidity and mortality worldwide. Infants less than 6 months of age are particularly vulnerable to development of severe disease following infection (1). Diseases associated with influenza virus infection in children include otitis media, pneumonia, myositis, and croup. While oseltamivir (Tamiflu), one of the two FDA-approved anti-influenza drugs, can be used in infants aged 2 weeks and older, concerns exist due to the potential for adverse effects, drug resistance, and limited effectiveness in young infants (2).

Currently, there are three approved approaches for vaccination against influenza in the United States: intramuscular (i.m.) administration of inactivated influenza virus, intramuscular administration of recombinant hemagglutinin (HA) proteins, and intranasal administration of a live attenuated influenza virus (LAIV). The first is approved for use in individuals aged 6 months and older, the second for use in individuals aged 18 to 49 years, and the last for use in healthy individuals aged 2 to 49 years. Thus, none are approved for use in the vulnerable neonate population. While the lack of approval for the use of these vaccines in the very young may reflect some safety concerns, a principal factor is the poor immune responses elicited in human neonates (3, 4).

Previous studies, while limited, have shown that an initial dose of the trivalent influenza vaccine (TIV) is not capable of inducing seroconversion (as defined by a 4-fold increase in antibody titer) in infants less than 6 months of age, with the exception of one H3N2 virus strain (A/Mississippi/11/85, for which the conversion rate was 40% for reasons that are unknown) (3). This low responsiveness was not the result of maternal antibody, as all individuals had prevaccination titers of <1:8. A second dose resulted in seroconversion rates of 27 to 32% for H1N1 strains and heterogeneous responses against H3N2 strains (seroconversion rates, 17 to 93%; median rate, 32%). Not surprisingly, a correlation between age

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and the rate of conversion was observed, with older infants converting at a higher rate than younger infants (3). In a second study, in a group of 10- to 22-week old infants, conversion was assessed following completion of two doses of vaccine, with the conversion rates being reported to be 42 to 43% for H1N1 strains and 39 to 67% for H3N2 strains (4). For comparison, published studies assessing responses in older children reported that the percentage of individuals between 11 and 16 years of age with a 4-fold rise in titer was >90% after a single vaccination (5). Thus, infants respond poorly to the standard vaccine, even after multiple vaccinations.

The poor responsiveness of this population to vaccination is not surprising, given the significant body of literature demonstrating functional defects in the neonatal immune system (6–9). These defects span the innate and adaptive immune responses. With regard to the generation of adaptive immunity, dendritic cells (DCs) from neonates have been reported to produce small amounts of interleukin-12 (IL-12) and are impaired in their ability to upregulate costimulatory molecules, e.g., CD80 and CD86, following exposure to virus-derived signals (e.g., see reference 10). In addition, lung-resident DCs in neonates have been reported to have defects in their ability to effectively traffic and activate T cells following respiratory infection, and this is especially the case for $CD103^+$ DCs, which play a critical role in $CD8^+$ T cell priming (11).

In addition to the impaired function of DCs, T lymphocytes from neonates exhibit inherent defects in their ability to undergo activation and differentiation (12–14), including reduced levels of Lck and ZAP-70 (15), both of which are crucial for the initiation of signaling, and decreased AP-1-mediated transcription (16). Further, T cells in neonates have been reported to be impaired with regard to migration to the lung following infection (17). The combined deficiencies in DC maturation and T cell responsiveness are likely major contributors to the impaired T cell responses observed *in vivo* following infection or vaccination (11, 18–20). In addition to decreased responsiveness, data suggest a Th2 bias in the neonate (7). Studies in murine models suggest that this may be in part due to the IL-4-driven apoptosis of Th1 cells (21) as a result of the reduced production of IL-12 (22).

Antibody responses are also significantly hampered in neonates (for a review, see reference 6). Important contributors to the poor antibody response in infants are impaired accessory cells, i.e., follicular Th (T_{FH}) cells (23) and follicular DCs (24), as well as inherent defects in B cell survival and differentiation (25). A potential contributor to diminished survival and differentiation is the reduced expression of B cell maturation antigen (BCMA) and BAFF-R on neonate B cells (26). In addition, survival of plasmablasts and differentiation into long-lived antibody-secreting cells in the neonate are likely hampered by decreased levels of APRIL (27). Finally, neonates appear to have increases in regulatory B and T cell populations (28–32).

Recently, it was suggested that select vaccine-adjuvant combinations can allow induction of effective immune responses and protection in neonates (33). Accumulating data support the idea that Toll-like receptor (TLR) agonists are effective enhancers of vaccine-mediated responses (34, 35). Flagellin (flg) is a TLR5 agonist that has proven to be a potent adjuvant for the induction of antibody responses in a number of experimental animal models (for reviews, see references 36 and 37). Studies in mice showed that its inclusion in an inactivated influenza virus vaccine resulted in enhanced virus-specific antibody and increased protection (38).

Flagellin promotes adaptive immunity through multiple pathways. First, it can directly activate human dendritic cells (39). Second, flagellin provides a direct stimulatory signal to human T cells, promoting increases in both proliferation and cytokine production (40). In mouse models, it was reported to augment recruitment of T and B cells to secondary lymphoid sites (41). Finally, a recent study showed that TLR5 is expressed on activated B cells and plasmablasts and as such can directly promote antibody responses in mice (42). Thus, the action of flagellin is multifaceted, which may contribute to its promising effects in the context of multiple vaccination approaches.

At present, our understanding of the *in vivo* adjuvant effects of flagellin have been derived from studies of adult animals. Here, we tested the hypothesis that flagellin could serve as an effective adjuvant for vaccination of neonates, particularly for vaccination against influenza. For these studies, we established an infant nonhuman primate (NHP) model. This was critical, as the distribution and/or function of TLR5 in primates can differ from that in mice (39, 43). For example, flagellin does not directly stimulate murine T cells (43, 44). An additional critical aspect of this model is the significantly longer period of infancy in NHPs, which allows assessment of boosting strategies. Thus, the primate model offers many advantages for the optimal assessment of the action of flagellin in neonates.

The results of our studies show that inclusion of flagellin results in increased influenza virus-specific IgG following both vaccination and challenge of neonates. In addition, infants receiving flagellin-adjuvanted vaccine exhibit increased numbers of influenza virus-specific T cells following virus challenge. These differences were clinically relevant, as animals vaccinated with inactivated PR8 influenza virus (IPR8) plus flagellin exhibited decreased lung pathology following virus challenge. These results support flagellin as a beneficial adjuvant for use in newborns.

MATERIALS AND METHODS

Animals. The African green monkey (AGM) infants (Caribbean-origin *Chlorocebus aethiops sabaeus*) used in this study were housed at the Vervet Research Colony at the Wake Forest School of Medicine. Infants were removed from their mothers at 1 to 3 days of age and moved to the nursery. The infants were initially housed in incubators and subsequently moved to caging when they were capable of thermoregulating. Animal health was assessed by monitoring body weight, temperature, respiration rate, heart rate, food intake, and activity throughout the experiment. Animals were allowed to acclimate to the nursery for 3 days prior to receiving the vaccine. All animal protocols were approved by the Institutional Animal Care and Use Committee at Wake Forest University School of Medicine.

Influenza virus A/PR/8/34 (H1N1). Virus stocks were grown and titers (50% egg infectious doses [EID_{50}]) were determined in fertilized chicken eggs essentially as described previously (45). Stocks were diluted in phosphate-buffered saline (PBS), flash frozen, and stored at −80°C.

Production of flagellin and m229. Flagellin (FliC) from *Salmonella enterica* serovar Enteritidis was prepared as previously described (46). Briefly, *Escherichia coli* BL21(DE3) containing pet29a::*fliC* encoding wild-type flagellin or truncated pet29a::229 encoding only the biologically inactive hypervariable region of flagellin (inactive flagellin with a mutation at position 229 [m229]) (47) was grown at 37°C in LB medium containing kanamycin (25 μg/ml) to an optical density (OD) at 595 nm (OD_{595}) of approximately 0.8. IPTG (isopropyl-β-D-thiogalactopyranoside) was then added to a final concentration of 1 mM, and incubation was contin-

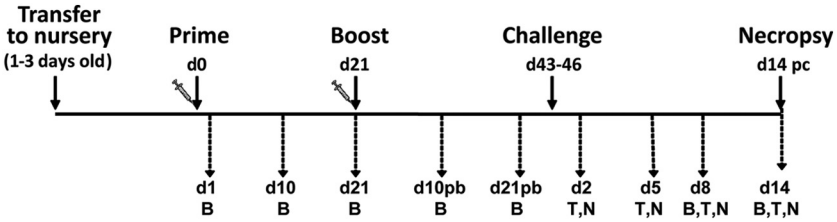

FIG 1 Experimental study design for vaccination and challenge. Neonatal African green monkeys (4 to 6 days old) were vaccinated (day 0 [d0]) and boosted 21 days later with IPR8 plus either flagellin or the inactive form of flagellin (m229). Control animals received PBS. Blood (B) was drawn on days 1, 10, and 21 after the initial vaccination and days 10 and 21 pb. At 23 to 26 days following the boost, the animals were challenged with live PR8 virus (10^{10} EID_{50}). On day 14 pc, the animals were necropsied. Tracheal tissue (T) and nasal swab (N) samples were acquired on days 2, 5, 8, and 14 pc, and blood was acquired on days 8 and 14 pc.

ued for an additional 5 h at 37°C. The cells were chilled on ice and harvested by centrifugation at 5,000 × *g* for 15 min. Cell-free lysates were prepared in 8 M urea, and the proteins were purified on Ni-nitrilotriacetic acid agarose (Qiagen) according to the manufacturer's protocol. Endotoxin and nucleic acids were removed using an Acrodisc Mustang Q capsule (Pall Corporation). The purified proteins were extensively dialyzed against PBS, pH 7.2. Protein concentrations were determined using a bicinchoninic acid protein assay (Pierce).

Vaccination. A/Puerto Rico/8/1934 (H1N1) (PR8), a mouse-adapted influenza A virus, was inactivated for use in vaccination by treatment with 0.74% formaldehyde overnight at 37°C. Virus was dialyzed against PBS and tested to ensure the absence of infectivity. At 4 to 6 days of age, infants were vaccinated with 45 μg of IPR8 in combination with 10 μg of either flg or m229 or with PBS as a control by intramuscular injection in the deltoid muscle (volume, 500 μl) (Fig. 1). Animals were boosted 21 days later. Six infants received IPR8 plus flg (IPR8-flg), five infants received IPR8 plus m229 (IPR8-m229), and three infants received PBS.

Virus challenge and sampling. On days 23 to 26 following the boost, the animals were sedated with 2 to 5% inhalant isoflurane. Animals received 1×10^{10} EID_{50} of strain PR8 divided equally between the intranasal (i.n.) and intratracheal (i.t.) routes: 0.25 ml i.t. and 0.25 ml i.n. (0.125 ml per nostril). On each sampling day, the animals were sedated. On days 8 and 14 postinfection, blood was collected by venipuncture and placed in tubes containing sodium heparin. Plasma was obtained by centrifugation, and peripheral blood mononuclear cells were subsequently isolated using the Isolymph reagent. Tracheal washes were performed on sedated animals on days 2, 5, 8, and 14 postinfection by inserting an endotracheal tube into the trachea, instilling 1.0 ml sterile PBS, and aspirating back. Due to the small volume of PBS used in the infants, 0.5 ml of PBS was used to wash out the endotracheal tube. Samples were centrifuged to remove cellular material, and bovine serum albumin (BSA) was added to a final concentration of 0.5%. Bronchoalveolar lavage (BAL) was performed at necropsy (day 14) using 5 ml of PBS. Samples were centrifuged to remove cellular material, and BSA was added to a final concentration of 0.5%.

Assessment of lung pathology. The lung was preserved in 10% neutral buffered formalin for at least 24 h, trimmed, embedded in paraffin, and processed for histology. Sections were cut at 6 μm and stained with hematoxylin and eosin. The slides were examined by light microscopy by an American College of Veterinary Pathologists board-certified veterinary pathologist in a blinded fashion and evaluated for the degree of inflammation and injury. Pathology assessment was based on interstitial and alveolar inflammatory cell infiltration and edema, pneumocyte hyperplasia, and bronchial degeneration and necrosis.

Quantitation of viral load. Viral RNA was extracted from the tracheal wash using a QIAamp viral RNA minikit (Qiagen). cDNA was synthesized from mRNA by reverse transcription (RT) using a SuperScript III reverse transcriptase kit (Invitrogen) and random primers (Invitrogen). For viral quantification, RNA primer-probe sets specific for H1N1 were used (BEI Resources). Quantitative RT-PCR (qRT-PCR) was performed using an Applied Biosystems 7500 real-time PCR system. EID_{50} were calculated on the basis of a standard curve generated using a stock of known EID_{50}. The total EID_{50} for the sample was calculated on the basis of the amount present in the sample volume used for the RT-PCR (140 μl) and by adjustment to the total volume used for the wash.

CRP measurement. The C-reactive protein (CRP) levels in plasma were assessed at 24 h postvaccination using a C-reactive protein enzyme-linked immunosorbent assay (ELISA) kit from Alpco Diagnostics per the manufacturer's instructions. The absorbance at 450 nm of the plate was read on a BioTek Elx800 absorbance microplate reader. Amounts were calculated on the basis of the standard curve generated using the control provided in the kit.

IL-6 measurement. The amount of IL-6 in plasma at 24 h postvaccination was measured using a human inflammatory cytokine cytometric bead array (CBA) kit from BD Biosciences per the manufacturer's protocol. Samples were acquired on a BD FACSCalibur system with CellQuest Pro software (Becton Dickinson). Analysis was performed using FCAP array software (version 3.0; SoftFlow/BD).

ELISA for detection of influenza virus-specific antibody. Nunc MaxiSorp ELISA plates were coated with 1 μg/well PR8 or 0.2 μg/well of recombinant HA (BEI Resources) in sodium carbonate/bicarbonate coating buffer (pH 9.5). Plates were blocked with 1× blocking buffer (10× blocking buffer; Sigma) plus 2% goat serum (Lampire Biologicals) and washed. The wash buffer used throughout the assay was PBS with 0.1% Tween 20. Plasma or respiratory samples were serially diluted in 1× blocking buffer. Wells without virus served as a negative control. Antibody specific for monkey IgG (Fitzgerald), IgM (LifeSpan Bioscience), or IgA (AbD Serotec) was used to detect bound antibody. IgG and IgM detection antibodies were directly conjugated to horseradish peroxidase (HRP). Anti-IgA antibody was biotinylated and was detected with streptavidin-HRP. Plates were developed with 3,3′,5,5′-tetramethylbenzidine dihydrochloride (Sigma), and the absorbance at 450 nm was read on a BioTek Elx800 absorbance microplate reader. The absorbance for each dilution was calculated by subtracting the OD value obtained for the corresponding non-virus-coated wells. The threshold titer was defined as the value that reached 3 times the assay background, i.e., the value for wells that received only sample diluent.

Neutralization assay. Heat-inactivated (56°C for 1 h) samples were serially diluted in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1× nonessential amino acids (NEAA), 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-mercaptoethanol, and 10% fetal bovine serum (FBS) in a sterile 96-well flat-bottom plate. Green fluorescent protein (GFP)-tagged PR8 (PR8-GFP; 7.5×10^6 EID_{50}; kindly provided by Adolfo Garcia-Sastre [48]) was added to each well, and the plate was incubated for 2 h at 37°C in 5% CO_2 to allow antibody binding. U937 cells (2×10^5) were then added to each well, and the plate was incubated overnight at 37°C. On the next morning, samples were acquired on a BD FACSCalibur system and analyzed with CellQuest Pro software (Becton Dickinson) to determine the percentage of U937 cells that were positive for GFP. Controls for each experiment consisted of U937 cells alone and U937 cells infected with the PR8 virus-GFP in the

absence of plasma. The maximal percent GFP was calculated for each experiment, and nonlinear regression (Prism software; GraphPad, La Jolla, CA) was used to determine the dilution at which 50% of the maximum amount of PR8-GFP-infected U937 cells was achieved.

Antibody-secreting cell ELISPOT assay. Enzyme-linked immunosorbent spot (ELISPOT) assay plates were coated with 1 μg/well of PR8 in PBS and stored overnight at 4°C. Control wells were coated with PBS. Plates were blocked with RPMI 1640 medium plus 10% FBS and 1% HEPES for 1 h. Cells were plated and incubated for 6 h at 37°C in 5% CO_2. Plates were washed with PBS plus 0.5% Tween 20. Spots were detected using anti-IgG-HRP (Fitzgerald) and True Blue substrate (KPL). Spots were analyzed by use of an ImmunoSpot analyzer (Cellular Technology Ltd.) and ImmunoSpot (version 3.2) software.

T cell ELISPOT assay. Dendritic cells were generated from bone marrow by culture in the presence of granulocyte-macrophage colony-stimulating factor and IL-4 for 6 days. Successful differentiation was assessed by flow cytometric staining for CD11c. DCs were infected with GFP-PR8 virus (48). Infected or mock-infected DCs were cocultured with autologous cells for 48 h in ELISPOT assay plates coated with anti-gamma interferon (anti-IFN-γ; GZ-4) or anti-IL-4 (IL-4-I) capture antibody (Mabtech). Following incubation, spots were detected using biotin-conjugated anti-IFN-γ (7-B6) or anti-IL-4 (IL-4-II) detection antibody, streptavidin-HRP-conjugated antibody, and True Blue substrate solution. Spots were analyzed by use of an ImmunoSpot analyzer (Cellular Technology Ltd.) and ImmunoSpot (version 3.2) software. For T cell fractionation experiments, $CD8\beta^+$ cells were isolated from total splenocytes using anti-human CD8β-phycoerythrin (PE) antibody (clone 2ST8.5H7; Beckman Coulter) and anti-PE microbeads (Miltenyi Biotec).

Statistical analysis. For continuous outcomes, groups were compared using 2-sample *t* tests (if the treatment group had two levels) or analysis of variance (ANOVA) models (if there were three or more levels for the treatment group), with specific contrasts being defined to compare pairs of groups when appropriate. If outcome data were not normally distributed, logarithmic transformations were used prior to the analyses. For analyses that included repeated measures (i.e., for IgG), a repeated-measures mixed model was fit, with the primate being considered a random effect in the model and treatment group and day being considered fixed effects. The treatment group-by-day interaction was examined first in these models, and if it was found to be nonsignificant, then that term was removed. Comparisons between pairs of groups or on particular days were performed within these mixed models if the overall group or day effects were found to be significant. Data were analyzed using Prism (version 5) software (GraphPad, La Jolla, CA) or SAS (version 9.3) software.

RESULTS

Flagellin promotes a mild inflammatory response in infant AGMs when added to the inactivated influenza virus vaccine. The goal of adjuvants is to promote an adaptive immune response in the absence of deleterious side effects. Innate signals elicited through engagement of TLR, which was the approach used in our study, can robustly enhance this process. Infant AGMs were vaccinated at 4 to 6 days of age, as outlined in Fig. 1. Infants received inactivated PR8 in combination with either wild-type flagellin (IPR8-flg) or inactive flagellin (IPR8-m229). Control animals received PBS. To assess safety and immune stimulation as a result of vaccination in the presence of flagellin, infants were monitored for changes in temperature, respiration, heart rate, and overall health every 4 h following receipt of the vaccine. No consistent change in any of these health indicators was observed (data not shown).

Blood was drawn at 24 h postvaccination to assess C-reactive protein (CRP) and IL-6 levels as an indicator of systemic flg-mediated immune activation/inflammation. We observed a significant increase in CRP levels at 24 h postvaccination in animals receiving the flg-adjuvanted vaccine compared with those in animals receiving the m229-adjuvanted vaccine (Fig. 2A), with the overall ANOVA *P* value for the difference among the three groups being equal to 0.0021 and with pairwise comparisons of the groups receiving IPR8-flg and IPR8-m229 with the group receiving PBS each having *P* values of less than 0.003. However, while the CRP level was elevated, this level of CRP is modest and would not be considered clinically problematic (49). The level of flagellin-induced IL-6 production was heterogeneous among the infants, with three of the six animals exhibiting increases at 24 h postvaccination (Fig. 2B). Vaccinated infants did not exhibit increases in IL-1β, IL-8, IL-10, tumor necrosis factor alpha, or IL-12 levels (data not shown). These findings suggest that these vaccines are not associated with adverse events in these neonates.

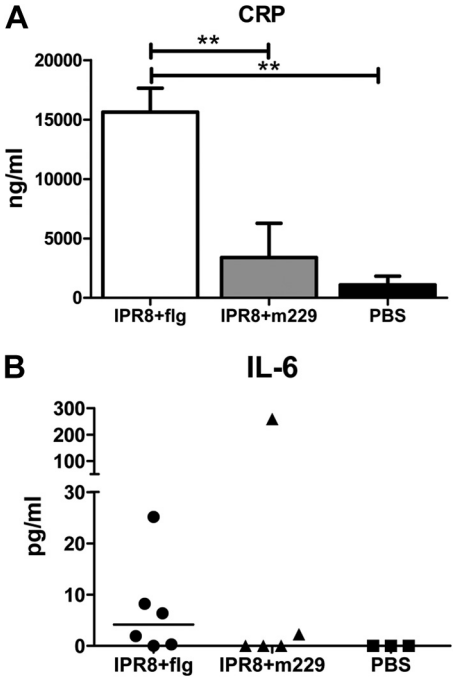

FIG 2 CRP levels are increased in animals vaccinated with IPR8-flagellin. CRP (A) and IL-6 (B) levels in the circulation were assessed at 24 h following vaccination. The presence of flagellin resulted in significantly increased levels of CRP in vaccinated infants. IL-6 levels did not differ significantly among the vaccinated groups. Significance was determined by ANOVA. **, $P < 0.01$.

Inclusion of flagellin during influenza vaccination enhances systemic humoral immune responses in infant AGMs. Production of virus-specific antibody was assessed at days 10 and 21 after the primary vaccination and at the same time points following the boost. The level of antibody detected at day 1 following primary vaccination served as a baseline. As shown in Fig. 3A, the presence of flagellin did not affect the level of virus-specific IgG generated as a result of primary vaccination (days 10 and 21). However, infants receiving flagellin-adjuvanted vaccine exhibited significantly increased levels of PR8-specific IgG on day 10 following the boost compared to infants vaccinated with IPR8-m229. Of note, one animal that received IPR8-m229 had a markedly heightened response compared to that of the other infants (even those that were vaccinated with the flagellin-adjuvanted vaccine). Outlier analysis revealed that the response in this animal was significantly different from that in the remainder of the animals in the group. The reason for the unusually robust response is not known but may reflect

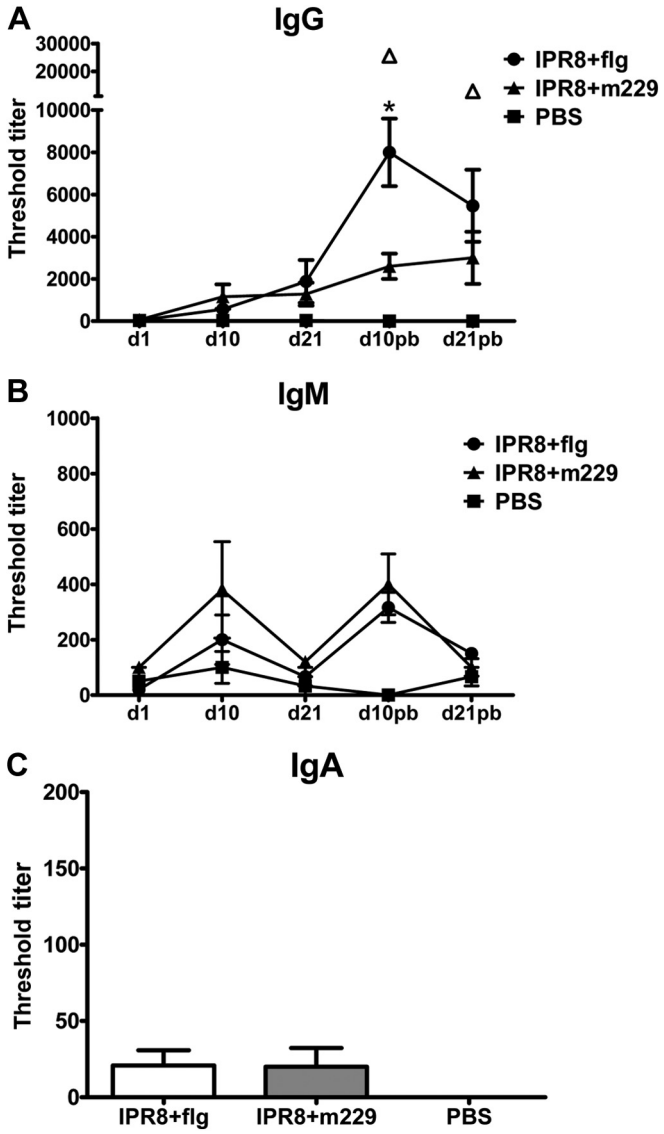

FIG 3 Inclusion of flagellin during vaccination results in an increase in influenza virus-specific antibody selectively following the boost. (A and B) The levels of PR8-specific IgG (A) and IgM (B) in plasma were measured by ELISA on days 10 and 21 following prime or boost. (C) The level of PR8-specific IgA in plasma was measured at day 10 pb. The threshold titer, defined as the dilution at which the OD_{450} reached three times the assay background, was calculated for each animal. Averaged data are shown. The influenza virus-specific IgG responses at 10 days following the boost observed in infants vaccinated with IPR8-flg were significantly increased compared to those observed in infants vaccinated with IPR8-m229. No differences in IgA or IgM responses were detected. Significance was determined using a repeated-measures-mixed-model fit. *, $P < 0.05$.

genetic factors. The atypical response in this individual is reminiscent of the variability in responsiveness that is observed in the human population, in that sporadic individuals can make an unusually robust response (e.g., see references 50 and 51). Inclusion of the value for this outlier animal in the average revealed the response in this animal to be significantly different than that of the remainder of the group. Thus, it was excluded in the remaining data sets. The repeated-measures-mixed-model fit for these data showed that there was a significant group-by-day interaction ($P = 0.046$). Groups were compared at each day to determine where differences existed. These tests determined that the responses to IPR8-flg and IPR8-m229 were significantly different by day 10 postboost (pb) ($P = 0.03$). Unexpectedly, the level of virus-specific IgG did not continue to increase through day 21. PBS-injected infants showed no detectable level of circulating PR8-specific IgG at any time point.

The virus-specific IgM responses in the infants were also assessed. In contrast to what we observed for IgG, while vaccination induced a modest increase in IgM compared to that in the nonvaccinated control group, there was no discernible difference between the two groups of vaccinated animals (Fig. 3B). Thus, the enhancing effects of flagellin are selective for IgG.

We also assessed the production of IgA following vaccination. Not surprisingly, given that the vaccine was delivered i.m., virus-specific IgA levels in plasma were minimal at day 10 pb, the time point at which maximal virus-specific IgG antibody was detected (Fig. 3C). Collectively, these data show that the inclusion of flagellin during vaccination significantly enhanced the virus-specific IgG immune responses in AGM neonates following boost.

An important attribute of protective influenza virus-specific antibody is the ability to prevent virus infection. To probe the potential for vaccine-generated antibody to function in this capacity, we assessed the ability of antibody generated as a result of vaccination to inhibit infection of tissue culture cells by a GFP-expressing influenza virus (48). Using this approach, we determined the half-maximal (50%) inhibitory concentration (IC_{50}), which was defined as the dilution of plasma that inhibited infectivity by 50% compared to the level of infection in the absence of plasma. This approach was chosen, as it is a direct measure of the ability of the antibody to prevent infection. This assay was validated against the standard hemagglutination inhibition (HI) assay. Plasma from an independent group of African green monkeys that were 14 days postinfection with influenza virus was assessed for the presence of neutralizing antibody using a standard HI assay or the flow cytometry-based assay. A total of 2.5×10^5 EID_{50} was used for the HI assay and 7.5×10^6 EID_{50} was used for the flow cytometry-based assay. In general, these two assays provided similar relative results, although absolute titers were lower in the flow cytometry-based assay, likely due to the larger amount of virus used (Fig. 4C).

Surprisingly, at day 10 pb neutralization titers were not significantly different between animals receiving IPR8-flg and animals receiving IPR8-m229 (Fig. 4A), even though total influenza virus-specific IgG levels were higher in IPR8-flg-vaccinated infants. This suggests that the increase in total influenza virus-specific IgG measured at day 10 pb was predominantly nonneutralizing antibody. Interestingly, neutralizing antibody titers remained relatively constant in IPR8-flg-vaccinated animals between days 10 and 21 pb (Fig. 4A). This contrasts with the findings for IPR8-m229-vaccinated animals, where a significant decrease was observed between days 10 and 21 pb (Fig. 4B). The sustained neutralizing titer in animals vaccinated in the presence of flagellin is consistent with the preferential retention of cells producing neutralizing antibody. Alternatively, the continued maturation of the response that includes the preferential generation of cells that produce neutralizing antibody could account for this finding.

The presence of flagellin does not promote the generation of IgG antibody with increased cross-strain recognition. The ability to induce antibody that is capable of recognizing heterologous

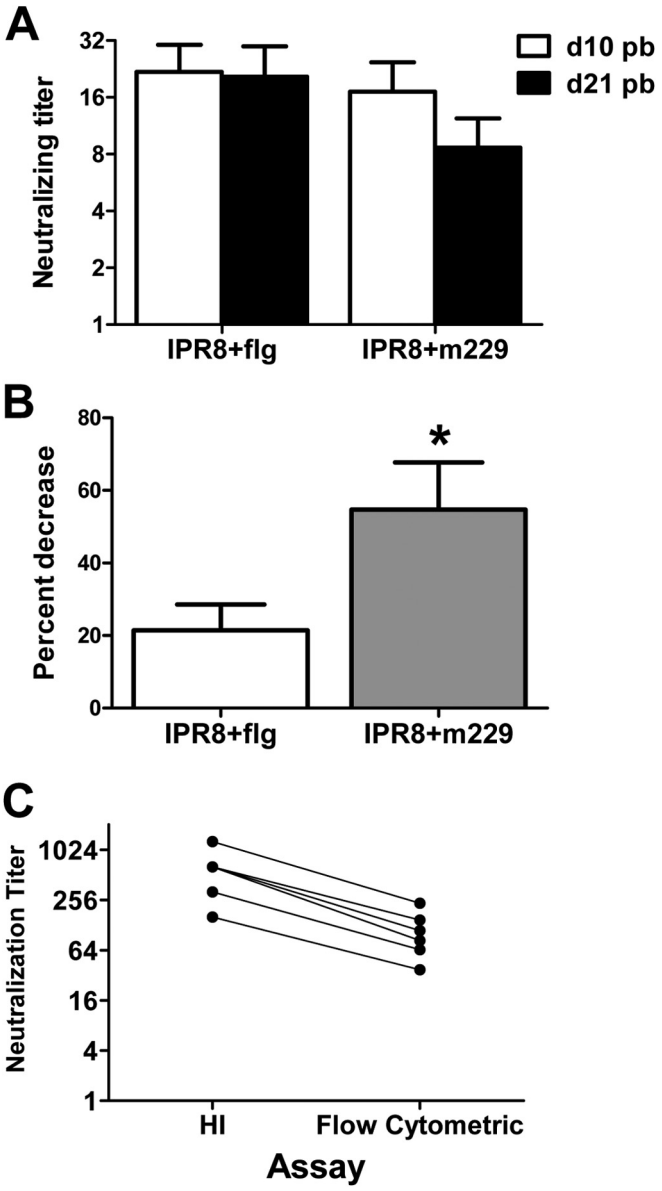

FIG 4 Inclusion of flagellin during vaccination results in a sustained neutralizing antibody response at late times postboost. (A) Neutralizing antibody titers in plasma were determined on days 10 and 21 pb by measuring the inhibition of infection of U937 cells by a GFP-expressing PR8 virus. The neutralization titer was defined as the half-maximal (50%) inhibitory concentration (IC_{50}), i.e., the dilution factor at which 50% infectivity was blocked. (B) The average percent decrease in the IC_{50} between day 10 and day 21 for each animal following the boost is shown. Significance was assessed using Student's *t* test. *, $P < 0.05$. (C) Comparison of the neutralizing antibody titers obtained using HI and a flow cytometry-based assay. Plasma from an independent group of influenza virus-infected African green monkeys was tested. PR8-GFP virus was used at 2.5×10^5 EID_{50} and 7.5×10^6 EID_{50} for the HI and the flow cytometry-based assay, respectively. A higher level of virus was used for the flow cytometry-based assay to promote infection of the majority of indicator cells. Although absolute titers were lower in the flow cytometry-based assay, likely due to the larger amount of virus used, the relative relationships were similar.

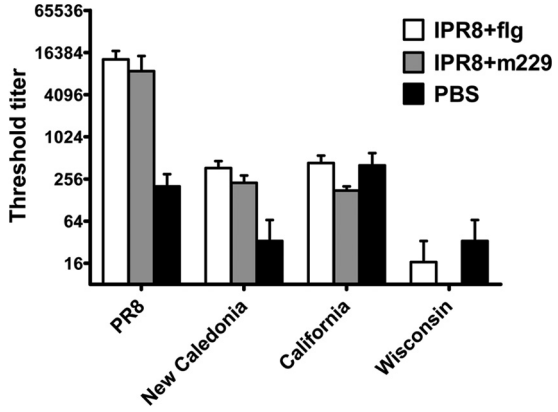

FIG 5 Antibody responses generated following vaccination in the presence or absence of flagellin have similar patterns of cross-reactivity. Plasma obtained from infants at day 21 pb was assessed by ELISA for the presence of IgG antibodies capable of recognizing the HA molecule from A/New Caledonia/20/1999 (H1N1), A/California/07/2009 (H1N1) pdm09, and A/Wisconsin/67/2005 (H3N2). No significant difference in the capacity to recognize heterologous strains was detected between animals that were vaccinated in the presence of flg and animals that were vaccinated in the presence of m229.

strains of influenza virus is a highly desirable attribute of a vaccine. To determine whether the presence of flagellin impacted this capacity, we assessed the recognition of A/New Caledonia/20/1999 (H1N1), A/California/07/2009 (H1N1) pdm09, and A/Wisconsin/67/2005 (H3N2). We chose to utilize plasma samples from infants at day 21 pb, as that amount of time allowed the longest period for maturation of the antibody response. Overall, there was limited cross-strain recognition (Fig. 5). While there was modest recognition of A/New Caledonia/20/1999, when evaluated for differential recognition by animals vaccinated in the presence or absence of flagellin, none was observed. There was no apparent vaccine-induced recognition of A/California/07/2009 pdm09 or A/Wisconsin/67/2005. Together, these data show that the presence of flagellin does not promote the generation of IgG antibodies capable of cross-strain recognition.

Virus-specific IgG levels are higher after virus challenge in AGM infants receiving flagellin-adjuvanted vaccine than animals vaccinated in the presence of m229. The ultimate goal of vaccination is to establish an immune response that provides enhanced protection following pathogen exposure. This is facilitated by preexisting immunity as well as the capacity to mount a robust and rapid response to infection. To assess the ability of the vaccinated NHP neonates to respond to pathogen challenge, infants were infected with PR8 virus at 23 to 26 days following the boost. PR8-specific IgG levels in the circulation and in the respiratory tract (trachea) were assessed.

Analysis of systemic antibody revealed a highly significant enhancement of the recall response in animals immunized with flagellin-adjuvanted vaccine compared to that in animals receiving m229 (Fig. 6A). For this comparison, the log2-transformed plasma IgG levels were compared using a one-way ANOVA, and the overall test showed a large difference among groups at day 8 ($P < 0.001$). Each of the three pairwise comparisons between the groups was also statistically significant on the basis of contrasts performed between groups ($P < 0.001$ for the group receiving PBS versus the group receiving IPR8-flg, $P = 0.002$ for the group receiving PBS versus the group receiving IPR8-m229, and $P = 0.004$ for the group receiving IPR8-m229 versus the group receiving IPR8-flg). On day 8 postchallenge (pc), PR8-specific IgG plasma responses in infants vaccinated with IPR8-flg were, on average, 5.6-fold higher than those in infants vaccinated with IPR8-m229.

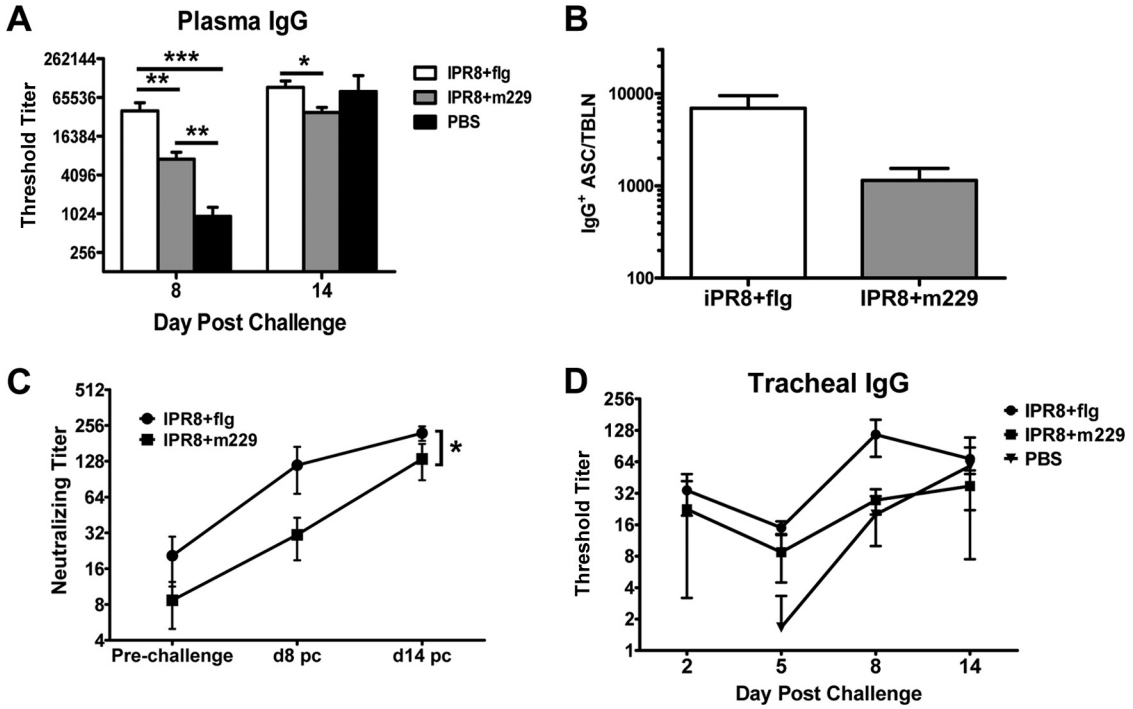

FIG 6 Inclusion of flagellin during vaccination results in an increase in virus-specific total and neutralizing antibody titers following challenge. (A) The influenza virus-specific IgG antibody titer was measured in plasma at day 8 and day 14 pc. (B) Influenza virus-specific IgG antibody-secreting cells (ASCs) in the draining TBLNs were quantified at day 14 pc by ELISPOT assay. (C) The neutralizing antibody titer in plasma was measured at days 8 and 14 pc. (D) Analysis of influenza virus-specific IgG in the trachea at days 2, 5, 8, and 14 postinfection. In panels A, B, and D, significance was determined by a one-way ANOVA. For panel C we fitted a repeated-measures-mixed-model ANOVA examining the neutralizing titer (log transformed) as the outcome and day, group, and day-by-group effects in the model. The day-by-group interaction was not significant. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.

At day 14 pc, plasma antibody levels in infants vaccinated with IPR8-flg continued to be significantly higher than those in animals vaccinated with IPR8-m229. The increase in antibody levels following challenge was associated with an increase in antibody-secreting cells in the draining tracheobronchial lymph nodes (TBLNs), although this did not reach statistical significance (Fig. 6B). The presence of neutralizing antibody was also assessed. The data in Fig. 6C show that IPR8-flg-vaccinated animals exhibited a significantly higher level of neutralizing antibody following challenge than their counterparts vaccinated in the presence of m229. When PR8-specific IgG in the trachea was examined, a correlative trend toward increased amounts of virus-specific antibody in the trachea in infants vaccinated in the presence of flg was observed at day 8 (Fig. 6D).

Vaccination with flagellin promotes robust T cell recall responses. Influenza virus-specific T cells play a critical role in the clearance of influenza virus (52). Thus, we assessed the T cell response present in vaccinated infants following challenge. IL-4 and IFN-γ production was assessed by ELISPOT assay, as this approach provides a highly sensitive readout for antigen-specific cells. Autologous DCs were generated from bone marrow obtained at necropsy. Differentiated DCs were infected with the PR8-GFP virus, which allowed assessment to ensure that efficient infection had occurred. Preliminary studies established that this approach resulted in the stimulation of both $CD4^+$ and $CD8^+$ T cells.

The postchallenge T cell responses in IPR8-flg-vaccinated animals were significantly increased for both IFN-γ- and IL-4-producing virus-specific cells in the spleen (Fig. 7A). There was also a trend toward increased influenza virus-specific IFN-γ-producing cells in the lungs in animals vaccinated with IPR8-flg, although interpretation was complicated by the high variability in IPR8-m229-vaccinated animals (Fig. 7B). Analysis of samples from a single late time point is an unavoidable limitation in the assessment of T cell responses in this tissue, as it is performed at necropsy. Assessment of samples obtained at alternative time points would be required to know how the early recall responses in the lungs of IPR8-flg-vaccinated animals versus the lungs of IPR8-m229-vaccinated animals differ.

We were interested to determine how the production of cytokine was distributed among $CD4^+$ and $CD8^+$ cells. To address this question, we developed an isolation approach that did not depend on CD4 to separate these two populations. This was necessitated by the understanding that $CD4^+$ T cells in AGMs can downregulate CD4 and concurrently upregulate CD8α following activation, resulting in a CD4-low/negative CD8α-dim population (53, 54). CD8β was used to isolated $CD8^+$ cells by magnetic separation. In order to determine how IFN-γ and IL-4 production was distributed among these populations, we separated splenocytes into $CD8\beta^+$ and $CD8\beta^-$ populations. The $CD8\beta^-$ population contained $CD4^+$ T cells regardless of the activation state. Isolated populations were stimulated with influenza virus-infected autologous bone marrow-derived DCs. The data in Fig. 7C are consistent with a flagellin-mediated increase in both $CD4^+$ and $CD8^+$ cytokine-producing effector cells, although significance was achieved only for IL-4-producing $CD4^+$ cells.

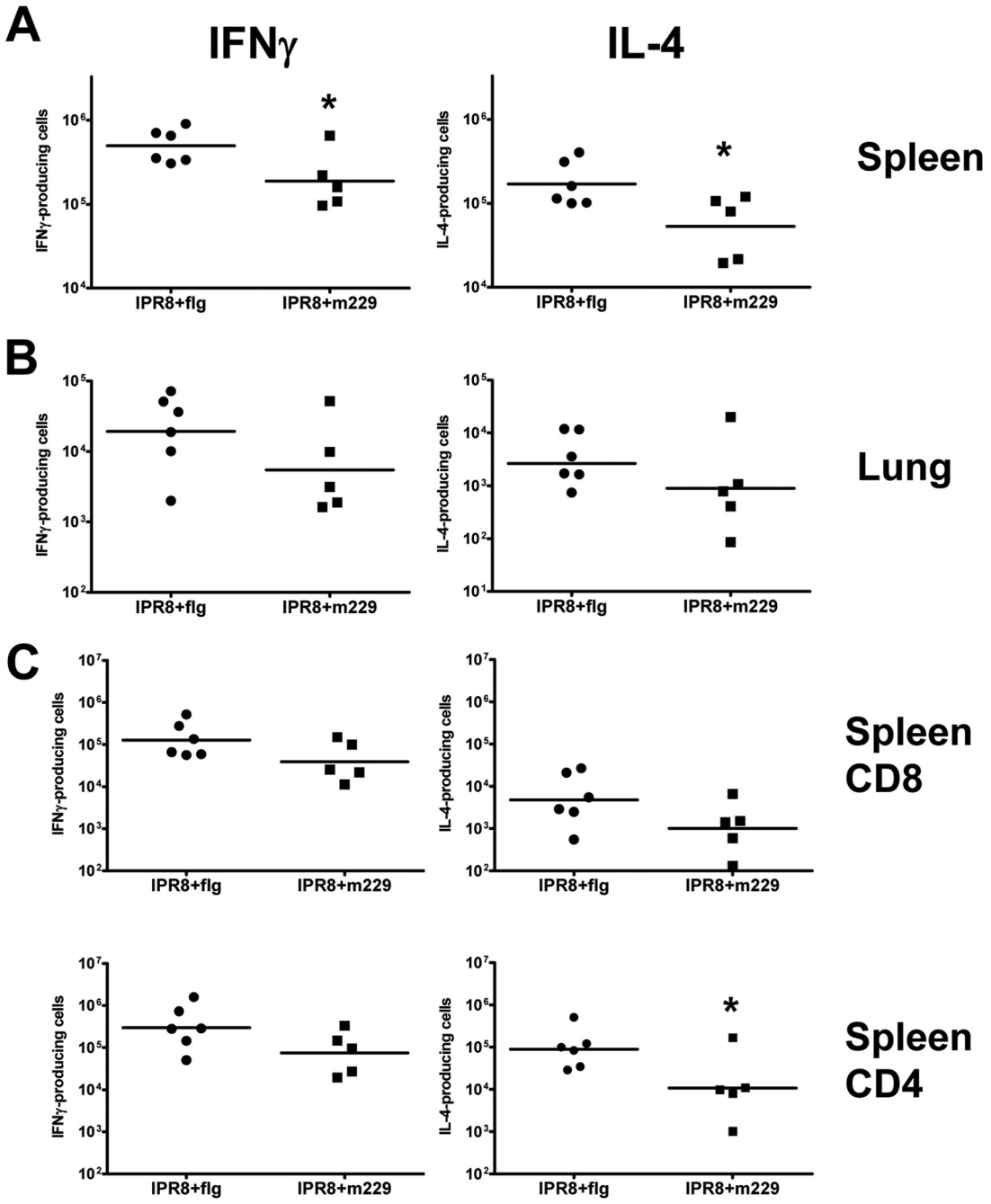

FIG 7 Infants vaccinated with IPR8-flg have an increased number of influenza virus-specific T cells after challenge. IFN-γ- and IL-4-producing influenza virus-specific T cells in the spleen (A) and lung (B) were measured by ELISPOT assay. The number of both IFN-γ- and IL-4-producing cells was significantly increased in the spleens of infants vaccinated with IPR8-flg compared to the number in the spleens of infants vaccinated with IPR8-m229. (C) Splenic $CD8\beta^+$ (CD8 effector) and $CD8\beta^-$ (CD4 effector) T cells were isolated from the spleen and stimulated in the presence of autologous DCs exposed to influenza virus. IL-4- or IFN-γ-producing cells were detected by ELISPOT assay. The results for individual animals (individual symbols) and the geometric means (bars) are shown. Significance was assessed using a Student *t* test. *, $P < 0.05$.

Flagellin-adjuvanted animals show reduced lung pathology and increased viral clearance after challenge with live PR8 influenza virus. In order to investigate whether inclusion of flagellin can result in improved protection, we assessed the viral load and lung pathology in vaccinated and nonvaccinated animals. The viral load (EID_{50}) was calculated on the basis of a standard curve generated following RT-PCR with infectious virus (Fig. 8A, right). Infants vaccinated with IPR8-flg had significantly reduced levels of virus in the nasal tract at day 2 postinfection compared to infants that received IPR8-m229 (Fig. 8A). Interestingly, at this time point infants vaccinated with IPR8-m229 appeared to have higher levels of virus than animals treated with PBS, perhaps suggestive of an enhancing effect of the vaccine-induced antibodies on infectivity when adjuvant was not present. While the average level of virus

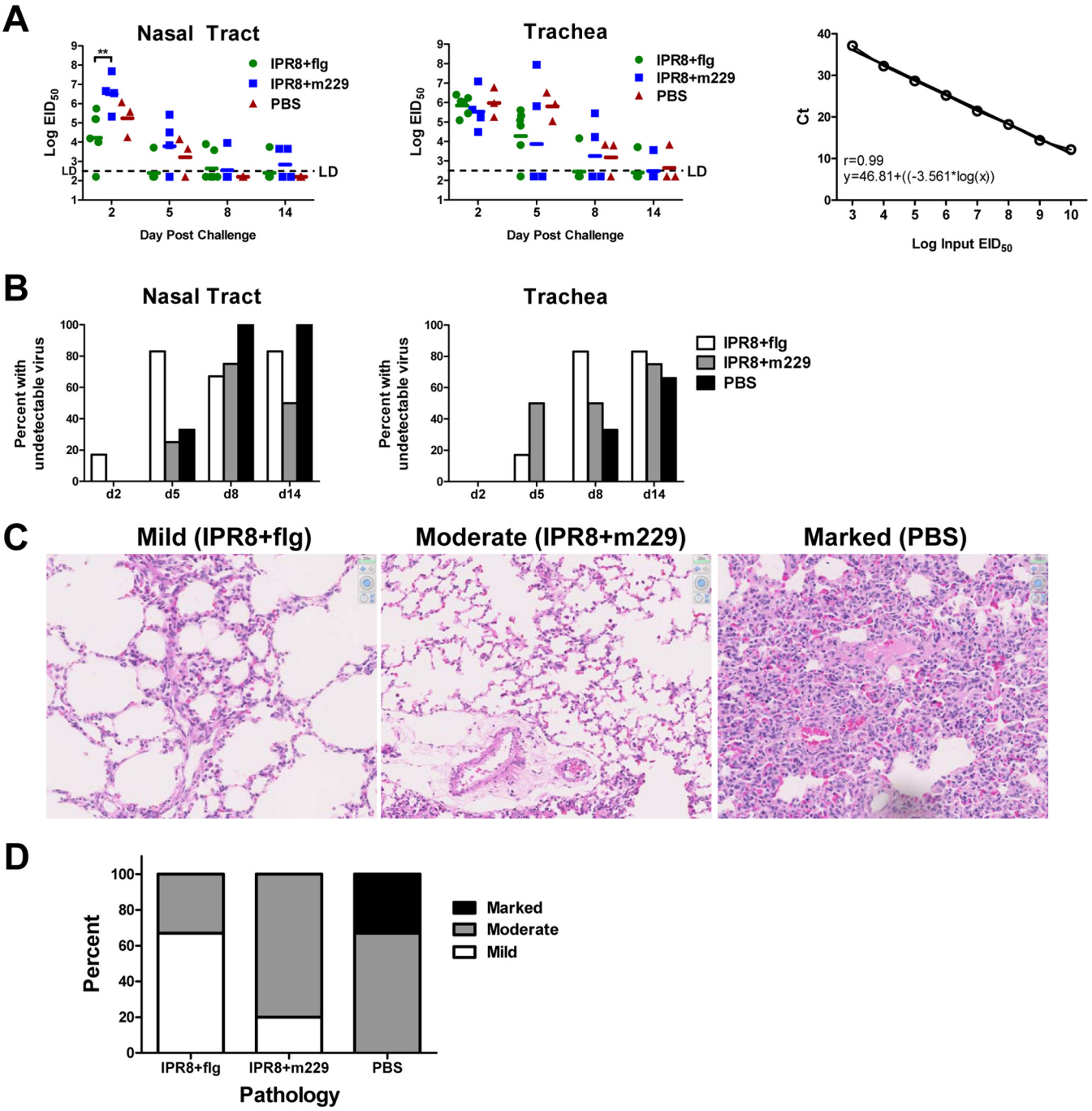

FIG 8 The presence of flagellin induces influenza virus-specific immune responses that are capable of increasing virus clearance and decreasing disease. (A) The virus load in the nasal tract (swabbing) and trachea (wash) was measured following virus challenge of vaccinated animals. At early times following challenge, the virus titers in the nasal tract of IPR8-flg-vaccinated infants were reduced compared to those in IPR8-m229-vaccinated animals. (B) Percentage of animals in which virus was undetectable in the nasal tract and trachea at each indicated time following challenge. (C) Representative sections from the lungs of challenged animals following vaccination with IPR8-flg, IPR8-m229, or PBS are shown. (D) The severity of the lung pathology was determined in a blinded fashion by a board-certified pathologist. Pathology was assigned as mild, moderate, or marked for each animal. Whereas the majority of animals vaccinated with IPR8-m229 had moderate lung pathology, the majority of animals receiving IPR8-flg exhibited mild disease.

was decreased in IPR8-flg-vaccinated infants compared to the level of virus in animals treated with PBS, this did not reach statistical significance. We did not observe significant differences in the average virus burden in the tracheal washes.

Admittedly, this analysis was challenging, as there was substantial variability among the animals. Thus, as another method to assess the control of virus following challenge, we scored animals as positive or negative according to whether virus was detected in the nasal tract and trachea at each time point assessed. This analysis showed that the proportion of animals that were negative for

virus in the nasal tract at day 2 and day 5 was greatly increased for IPR8-flg-vaccinated infants compared to infants that received IPR8-m229 (Fig. 8B). Surprisingly, at later times, infants vaccinated with IPR8-m229 and, to a lesser extent, infants vaccinated with IPR8-flg seemed to have a reduced ability to completely clear virus from the nasal tract compared to nonvaccinated animals. This was an unexpected finding, given that at early times virus was undetectable in a large number of the IPR8-flg-vaccinated infants. Additional studies are necessary to fully define the potential for upper airway persistence in a small percentage of infants. The lower respiratory tract (trachea) was also evaluated for the presence of detectable virus over the course of the infection. Infants vaccinated with IPR-flg showed improved clearance of virus by day 8 compared to the clearance observed for infants that received the m229-adjuvanted vaccine.

In order to address whether the presence of flagellin during vaccination reduced the severity of virus-mediated disease, lung pathology was evaluated in a blinded fashion on day 14 postchallenge. A representative section from each group is shown in Fig. 8C. Mild inflammation was characterized by a slight thickening of the alveolar walls and the minimal presence of intra-alveolar inflammatory cells. Moderate inflammation was characterized by modest thickening of the alveolar walls, perivascular edema, and regular intra-alveolar cells. Intra-alveolar macrophages were regularly present. In addition, at a higher magnification, closely spaced cuboidal epithelial cells, characteristic of type 2 pneumocyte hyperplasia, lining the alveolar walls were occasionally apparent (data not shown). Severe interstitial pneumonia was characterized by diffuse hypercellularity, obscuring the small airways.

Whereas the majority of animals vaccinated with IPR8-m229 had moderate lung pathology, the majority of animals that received IPR8-flg exhibited mild disease (Fig. 8D). All nonvaccinated infants developed either moderate or marked lung disease. Taken together, these data suggest that AGM infants vaccinated with IPR8-flg more effectively clear virus at early time points postchallenge and display disease scores milder than those for animals that received IPR8-m229 or PBS.

DISCUSSION

There is a clear need for the development of vaccine approaches against influenza that are effective in young infants. In addition to overcoming the immune deficiencies associated with the neonatal immune system, efforts to achieve this goal are hampered by limitations in appropriate animal models that closely mimic human infants in immune development as well as in the expression/responsiveness of molecules that sense the pathogen and immunostimulatory agents. In this study, we have utilized a novel model for the development of influenza vaccines that can be effective in neonates. We propose that infant NHPs are a highly advantageous model as, in contrast to the commonly used mouse model, (i) at birth the immune system of NHPs more closely mirrors that of humans (55), (ii) there is a high degree of similarity of NHPs to humans with regard to the distribution and responsiveness of TLR receptors (56), and (iii) the NHP lung shares strong similarities with the human lung in structure and development (57).

In our studies, we found that inclusion of flagellin in an inactivated influenza virus vaccine resulted in superior recall responses (both antibody and T cell responses) following infection. We propose that this higher early IgG response has the potential to limit the spread of influenza and, as a result, reduce the incidence of disease in the lower respiratory tract. Such a model is supported by our finding that animals vaccinated in the presence of flagellin exhibited lower lung pathology.

The ability of flagellin to serve as an effective adjuvant in adults has been assessed in experimental vaccines against a broad range of pathogens, including *Clostridium*, influenza virus, *Plasmodium*, *Streptococcus*, *Yersinia*, HIV, West Nile virus, *Salmonella*, *Enterococcus*, *Pseudomonas*, *Campylobacter*, *Helicobacter*, and *Burkholderia* (for a review, see reference 37). In adult animals, flagellin has been shown to increase both antibody and T cell responses (for example, see references 38 and 58 to 62). The promising results in experimental models (e.g., see reference 61) have led to the evaluation of flagellin in phase 1 clinical trials, including as part of an F1/V fusion protein to protect against plague (http://clinicaltrials.gov/show/NCT01381744), as a fusion protein with influenza virus matrix protein 2 (63, 64), or with the influenza virus hemagglutinin protein (65, 66). Results from the studies of influenza virus fusion proteins demonstrate that these vaccines are safe and immunogenic in healthy adults (63–65) as well as in the elderly (66). While the efficacy of flagellin has not previously been assessed in neonates, a study with 4- to 6-month-old juvenile monkeys found that vaccination with a fusion protein containing flagellin and the *Pseudomonas aeruginosa* outer membrane proteins OprI and OprF resulted in a high-affinity IgG antibody that was capable of providing passive protection against *Pseudomonas aeruginosa* infection in a mouse model (62).

The most striking effect of flagellin in our studies with neonates was in promoting a rapid antibody recall response. This was apparent following boosting as well as challenge. Our initial rationale for choosing flagellin hinged on its capacity to promote a T cell response, given the known expression of TLR5 on primate T cells (67), with the hypothesis that this would support the antibody response. Further, there are data supporting the ability of flagellin to enhance the activation of T cells isolated from infants (40). Specifically, cord blood-derived $CD8^+$ T cells exhibited increased levels of proliferation and IFN-γ production when stimulated in the presence of flagellin (40). While it has not yet been reported, if flagellin engagement by neonatal $CD4^+$ T cells has a similar effect, which seems likely, given the expression of TLR5 on $CD4^+$ T cells (68), then we would predict that flagellin could support the generation of a Th response that promotes antibody production. Our results are consistent with the potential for flagellin to promote a T cell response, as evidenced by the increase in influenza virus-specific effector T cells observed following challenge in animals that were vaccinated in the presence of flagellin.

While naive and memory B cells have been reported to be negative for TLR5 expression (69, 70), flagellin was recently found to promote the generation and/or survival of short-lived plasma cells, and data suggest that TLR5 expression on these cells contributes to this effect (42). In addition, activated B cells were found to upregulate the expression of TLR5 (42). These results suggest that flagellin has the potential to modulate previously activated B cells as well as short-lived plasma cells. The selective expression of TLR5 on these two populations would align with the restriction of flagellin-mediated enhancement of the antibody response to the boost and challenge phases of our study. The effect at these times would also be in keeping with the limited time frame of flagellin persistence following vaccination (J. Bates and S. B. Mizel, unpublished data), as at the time of vaccine administration there would be TLR5-bearing virus-specific B cells and/or plasma cells that

could respond to this stimulatory signal. Effects on the short-lived plasma cells could also account for the unexpected finding that the flagellin-mediated increase in antibody at day 10 pb did not continue for a prolonged period, as at day 21 pb antibody levels were slightly decreased compared to those measured at day 10 pb. If flagellin is found to have direct effects on short-lived plasma cells, approaches that would promote the greater survival of these cells when influenza vaccine is used in combination with flagellin are avenues for exploration in optimizing the use of flagellin as an adjuvant for neonates.

The ability of flagellin to modulate immune responses in the neonate is interesting, given the recent study by Oh et al. reporting a role for microbiota-dependent TLR5 signaling in promoting responsiveness to TIV (42). The authors found that signals provided by gut commensals promoted the humoral response, increasing the quantity of antibody and the frequency of plasma cells. The flora of humans undergoes significant change through the first years of life, with adult-like populations being established at about 3 years of age (for a review, see reference 71). The composition of the gut flora has important consequences for vaccination, as evidenced in a study of human infants receiving an array of vaccines (72). It is tempting to speculate that the commensals present in the gut of neonates may provide suboptimal TLR5 signals, which may be a factor contributing to the poor vaccine responses in young infants. The presence of flagellin in the vaccine may compensate for the lack of an endogenous signal, thereby promoting a more robust antibody response following vaccination. More studies are needed to determine the extent to which the gut flora of neonates contributes to vaccine responsiveness and how adjuvants may overcome any defects associated with the microbiota of the neonate.

While the effects of flagellin with regard to enhancing recall responses are promising, the ability to further increase the level of total and neutralizing antibodies present following a boost is desirable. Recent data suggest that combined signaling through multiple TLRs may have synergistic effects (73–76). Thus, one approach to continued improvement of the vaccine strategy for neonates is the inclusion of additional TLR agonists. In addition to flagellin, a number of TLR agonists are currently being explored, and many of these have been clinically evaluated, e.g., poly(I·C) (a TLR3 ligand), monophosphoryl lipid A (a TLR4 ligand), imiquimod (a TLR7 ligand), and CpG (a TLR9 ligand) (77). Whether the presence of multiple TLR ligands would increase the response to vaccination in the context of the neonate remains to be tested.

In summary, our study using AGM neonates supports the continued exploration of flagellin as an effective adjuvant in the context of the neonate. This is especially true with regard to the induction of responses that can expand rapidly following a secondary antigen encounter (boost or infection). In our studies, we found no evidence of adverse effects, suggesting that flagellin is safe in this population. Future studies evaluating the mechanism through which flagellin exerts its effects on the recall response will move us closer to exploiting this adjuvant as a novel vaccine component for use in neonates.

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